Expression of an alfalfa (*Medicago sativa* L.) peroxidase gene in transgenic *Arabidopsis thaliana* enhances resistance to NaCl and H₂O₂

K. Teng¹, G.Z. Xiao², W.E. Guo¹, J.B. Yuan¹, J. Li³, Y.H. Chao¹ and L.B. Han¹

¹Turfgrass Research Institute, College of Forestry, Beijing Forestry University, Beijing, China

²The College of Horticulture and Garden, Yangtze University, Jingzhou, China

³Institute of Grassland Research, Chinese Academy of Agricultural Sciences, Hohhot, China

Corresponding authors: Y.H. Chao / L.B. Han

E-mail: chaoyuehui@163.com / hanliebao@163.com

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ABSTRACT. Peroxidases (PODs) are enzymes that play important roles in catalyzing the reduction of H₂O₂ and the oxidation of various substrates. They function in many different and important biological processes, such as defense mechanisms, immune responses, and pathogeny. The POD genes have been cloned and identified in many plants, but their function in alfalfa (*Medicago sativa* L.) is not known, to date. Based on the POD gene sequence (GenBank accession No. L36157.1), we cloned the POD gene in alfalfa, which was named *MsPOD*. *MsPOD* expression increased with increasing H₂O₂. The gene was expressed in all of the tissues, including the roots, stems, leaves, and flowers, particularly in stems and leaves under light/dark conditions. A subcellular analysis showed that *MsPOD* was localized outside the cells. Transgenic *Arabidopsis* with *MsPOD* exhibited increased resistance to H₂O₂ and NaCl. Moreover, POD activity in the transgenic plants was significantly higher than that in wild-type

Arabidopsis. These results show that *MsPOD* plays an important role in resistance to H_2O_2 and NaCl.

Key words: *Medicago sativa* L.; Transgenic *Arabidopsis thaliana*; Peroxidase; Abiotic stress

INTRODUCTION

Under natural conditions, plants are subject to abiotic and biotic stress. Plants produce reactive oxygen under normal growth conditions, and can be induced to produce a large quantity of reactive oxygen species (ROS) under different stressors that can damage plants. To decrease the damage caused by ROS, plants activate the enzymatic system, which mainly consists of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidases (APX), and peroxidase (POD). PODs (EC 1.11.1.7), a family of isozymes found in all plants, can catalyze the reduction of H_2O_2 via the transport of electrons to various donor molecules, including phenolic compounds, lignin precursors, and auxin (Hiraga et al., 2001; Passardi et al., 2005).

PODs play an important role in abiotic stress tolerance in various plant species, including rice (*Oryza sativa*) (Demiral and Turkan 2005), barley (*Hordeum uhulgare*) (Liang et al., 2003), tomato (*Solanum lycopersicum*) (Mittova et al., 2004), pepper (*Capsicum baccatum*) (Gonçalves et al., 2013), and Kentucky bluegrass (*Poa pratensis*) (Li et al., 2014). Some plant POD genes have been expressed using transgenic techniques. The phenotypes of these transgenic plants yield information about gene function, such as *TPX2* improves the germination rate of tomato (*Lycopersicon esculentum*) under NaCl stress (Amaya et al., 1999) and *swpa1* and *swpa4* increase the H_2O_2 -scavenging capacity of sweet potato (*Ipomoea batatas*) (Yun et al., 2000; Kim et al., 2008).

Due to the physiologically important roles of PODs in plants, they have become the subject of a broad range of biochemical and molecular biological studies. A considerable amount of research on POD genes has been conducted in *Arabidopsis*, rice, and tomato, and it is known that POD genes play different roles in plant growth and developmental regulation. Alfalfa (*Medicago sativa* L.) is one of the most important forage grasses in the world, and is worthy of genetic study in order to improve its quality. The function of POD in alfalfa is not well known, and few studies have been conducted on it. In particular, the role of alfalfa POD in stress responses and tolerances is unknown. A detailed analysis of alfalfa POD will provide useful information about the alfalfa genes that are involved in stress responses and tolerances. Therefore, the isolation and characterization of POD in alfalfa will provide new insights into the biochemistry, roles, and regulation of these genes in transgenic plants.

In this study, we cloned a POD gene from the alfalfa cultivar 'Zhongmu No.1' that was named *MsPOD*. In order to detect to which abiotic stressor *MsPOD* responds, we analyzed its expression in response to H_2O_2 , salt, abscisic acid (ABA), gibberellic acid (GA), salicylic acid (SA), and methyl jasmonate (MeJA). In addition, we measured the physiological traits of transgenic *MsPOD Arabidopsis* plants after treatment with salt and H_2O_2 . The results show that the salt and oxidative tolerance of transgenic *Arabidopsis* plants significantly increased compared with that of non-transgenic *Arabidopsis* plants, and indicate that *MsPOD* plays an important role in increasing plant tolerance to abiotic stressors.

MATERIAL AND METHODS

Plant material and growth conditions

The alfalfa seeds used in this study were obtained from the Chinese Academy of Agricultural Sciences. All of the *Arabidopsis thaliana* lines had a Col-0 background. Plants were grown in a controlled room at 22°C under long-day (LD) conditions (16 h light and 8 h dark). For growth on tissue culture plates, *Arabidopsis* seeds were sown on ½ Murashige and Skoog (MS) solid medium containing 1% sucrose and 0.8% agar, and incubated at 22°C under LD conditions.

Amplification of full length *MsPOD*

Total RNA was isolated from 10-day-old seedlings by the TRIzol method and then extensively pretreated with RNase-free DNase to eliminate any contaminating genomic DNA before use. First-strand synthesis was performed using PrimeScript™ Reverse Transcriptase (TAKARA, Japan). Based on the sequence of the POD gene in GenBank, a pair of gene-specific primers, MsPOD-f and MsPOD-r (Table 1), was designed to amplify the full-length cDNA of *MsPOD*. The PCR conditions were as follows: 30 s at 95°C, 30 s at 55°C, and 2 min at 72°C for 30 cycles. The PCR products were separated by electrophoresis on a 1% agarose gel stained with ethidium bromide and purified using a DNA gel extraction kit (Biomed, Beijing, China). The products were cloned into a pEASY®-T1 vector (TransGen, Beijing, China) and then transformed into *Escherichia coli* DH5α. Recombinant plasmids were sequenced by the Beijing Genomics Institution (Beijing, China).

Table 1. Primers used in the study.

Primer	Sequence (5'-3')	Purpose of primer
MsPOD-f	AATAATGAACCTCTTCTTCAATCTC	RT-PCR of <i>MsPOD</i>
MsPOD-r	CTCTAGAACACACTCTTTATTCTTA	
MPF	gggCTCGAGATGAACCTCTTCTTCAATC	Construct subcellular localization vector of <i>MsPOD</i>
MPR	cccACTAGTAGGTATGAGCTAACAATACC	
POD-1	CTCTTTTCTTTGAAGCTTTTAAGGC	qRT-PCR analysis of <i>MsPOD</i>
POD-2	ATCCTCTGACGACTCTCTGATGACT	
PODf	cccTCTAGAATGAACCTCTTCTTCAATC	Construct plant expression vector to transform <i>Arabidopsis</i>
PODr	cccCCCGGTATGAGCTAACAATACC	
UBQ-f	GCAGCAACCAACGAAGCAAGA	qRT-PCR of alfalfa <i>Ubiquitin</i> gene used as internal control
UBQ-r	CACCACGAAGACGTAGGACAAGG	

Bioinformatics analysis

The amino acid sequence for MsPOD was obtained by cDNA translation using DNAMAN 6.0 software, and related proteins were obtained by a BLAST analysis of the National Center for Biotechnology Information database. Phylogenetic analysis was conducted using MEGA 5.0 software by the neighbor-joining method, and a bootstrap analysis was obtained from 1000 replications (Tamura et al., 2011). The theoretical isoelectric point (pI) and molecular weight (Mw) were predicted using the Compute pI/Mw tool (http://web.expasy.org/compute_pi/), and potential signal peptide cleavage sites were identified using SignalP 4.1 Server (<http://www.cbs.dtu.dk/services/SignalP/>) (Petersen et al., 2011). To predict the subcellular localization of MsPOD, the protein sequence was analyzed using the PSORT program (<http://www.psort.org/>).

Subcellular localization of MsPOD

To ascertain the subcellular localization of MsPOD, two primers, MPF and MPR (Table 1), were designed to amplify the coding DNA sequence of *MsPOD*. The PCR product was digested with *Xba*I and *Sma*I and subcloned into the vector pA7-GFP, which was digested with the same enzymes. The MsPOD-GFP fusion construct was transformed into onion epidermal cells by particle bombardment, as described in the manual of the high-pressure gas gene gun (GJ-1000; Ningbo Scientz Biotechnology, China) used. After 16 h of dark treatment, the onion epidermal cells were visualized with a confocal laser-scanning microscope (TE2000-E; Nikon, Japan).

Quantitative real-time PCR

In order to investigate the expression patterns of *MsPOD* in alfalfa, quantitative RT-PCR reactions were conducted for the roots, stems, leaves, and flowers. *MsPOD* expression profiles were determined in 10-day-old alfalfa seedlings induced by 2 mM H₂O₂, 20 μM GA₃, 20 μM ABA, 3 mM MeJA, and 200 mM NaCl, over a period of 24 h. The gene-specific primers used were POD-1 and POD-2 (Table 1). The qRT-PCR was performed on 96-well blocks using an Applied Biosystems 7500 Real-Time PCR system with SYBR[®] Green I Master Mix in a volume of 25 μL. The two-step thermal cycling profile used was 15 s at 95°C and 1 min at 68°C. The alfalfa *Ubiquitin* gene was used as a control in order to normalize the amount of cDNA. Relative expression was calculated using the comparative ΔΔ threshold cycle method (Chao et al., 2009). All of the results are reported as means of at least three independent RNA extractions (including three technical replicates) with their corresponding standard deviations (SD).

Plasmid construction and *Arabidopsis* transformation

The PCR products were purified and cloned into a binary plasmid (pBI121) at the *Xba*I-*Sma*I sites, which generated the plant expression vector 35S_{pro}::*MsPOD*. The 35S_{pro}::*MsPOD* construct was transferred into wild-type (WT) *Arabidopsis* plants by the floral dip method (Clough and Bent 1998). Transgenic seeds were selected on ½ MS plates with 50 mg/L kanamycin.

β-glucuronidase (GUS) staining

Histochemical GUS staining in the transgenic plants was performed as described previously (Cervera 2005). Briefly, the plant materials were stained in GUS assay solution containing 1 mM 5-bromo-4-chloro-3-indole-β-D glucuronide, 1 mM ethylenediaminetetraacetic acid, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 0.1% Triton X-100 in 500 mM potassium phosphate buffer, pH 7.0. After washing the samples with ethanol, GUS images were taken with a Canon PowerShot G3 digital camera (Cannon Inc.).

Analysis of POD and CAT activity

Leaves from two-week-old transgenic and WT seedlings were harvested. POD and CAT activity was determined using the method of Maehly and Chance (Maehly and Chance 1954), with modifications. For the POD analysis, the reaction mix contained 50 μL of enzyme

extract, 1.42 mL 10 mM phosphate buffer, pH 7.0, and 25 μ L 20 mM guaiacol. The reaction started with the addition of 10 μ L 40 mM H_2O_2 . POD activity was assayed by recording the increase in absorbance at 470 nm for 1 min. For the CAT assay, the reaction mixture contained 100 μ L of extracted solution, 45 mM H_2O_2 , and 50 mM sodium phosphate buffer, pH 7.0. The reaction started with the addition of the enzyme solution. The decrease in absorbance at 240 nm was read every 10 s for 1 min.

RESULTS

Isolation of *MsPOD* and sequence analysis

The full-length cDNA of *MsPOD*, including a 1062-bp coding domain sequence that encodes a polypeptide with 353 amino acids (Figure 1A), was cloned from alfalfa using the primers MsPOD-f and MsPOD-r. Amino acid sequence analysis revealed that the theoretical pI/Mw was 5.76/38200.02. A potential signal peptide was found with a cleavage site between pos. 28 and 29 in the MsPOD protein. A phylogenetic comparison of the MsPOD protein and its orthologs in *Medicago truncatula*, *Pisum sativum*, *Lupinus albus*, *Glycine max*, *Phaseolus vulgaris*, *Ficus carica*, *Linum usitatissimum*, *Populus trichocarpa*, *Hevea brasiliensis*, *Corylus avellana*, *A. thaliana*, and *O. sativa* showed that MsPOD is most closely related to the ortholog from *M. truncatula* (Figure 1B).

Figure 1. Analysis of *MsPOD* and its expression patterns. **A.** *MsPOD* encodes a polypeptide of 353 amino acids. **B.** Phylogenetic analysis of *MsPOD* protein orthologs. Sources of the orthologous proteins are indicated in parentheses. Bootstrap values are provided to indicate reliability at each node. **C.** Expression levels of *MsPOD* in roots, stems, leaves, and flowers of wild-type (WT) alfalfa plants. **D–H.** *MsPOD* expression levels after treatment with 2 mM H₂O₂ (**D**), 20 μ M gibberellic acid (GA₃) (**E**), 200 mM NaCl (**F**), 20 μ M abscisic acid (ABA) (**G**), and 3 mM methyl jasmonate (MeJA) (**H**). Error bars represent SEs (N = 3).

***MsPOD* expression patterns**

MsPOD transcripts were detected in all of the alfalfa organs, including the roots, stems, leaves, and flowers. *MsPOD* expression was higher in the stems and leaves than in the roots and flowers (Figure 1C). The expression level increased when 2 mM H₂O₂ was added (Figure 1D). *MsPOD* transcript levels under H₂O₂ conditions were time-dependent. Expression decreased after treatment with 20 μM GA₃ (Figure 1E), but increased slightly when 200 mM NaCl was applied (Figure 1F). When the alfalfa was exposed to 20 μM ABA or 3 mM MeJA, *MsPOD* expression levels were not affected for the first 6 h but decreased drastically after 24 h (Figure 1G, H). The results show that *MsPOD* was drastically induced under H₂O₂ conditions, indicating that it is involved in oxidative stress.

Subcellular localization of MsPOD

The PSORT program indicated that the MsPOD protein may appear outside of plant cells. To determine the subcellular localization of the MsPOD protein, the construct MsPOD-GFP driven by a 35S promoter was applied to transform onion epidermal cells. The fusion protein MsPOD-GFP was localized to the extracellular space of onion epidermal cells (Figure 2), indicating that MsPOD is localized outside plant cells.

Figure 2. Subcellular localization of MsPOD in onion epidermal cells. Fluorescence due to an untargeted 35S_{pro}::GFP plasmid was found in the whole onion cell. Red magnification bar, 50 μm. Fluorescence due to an MsPOD-GFP fusion protein was strongly detected in the extracellular space of onion epidermal cells. White magnification bar, 100 μm.

Selection of transgenic *Arabidopsis*

The full-length *MsPOD* sequence was placed under the control of a CaMV-35S promoter. The resulting 35S_{pro}::*MsPOD* construct was successfully transformed into *A. thaliana*, and 11 putative transgenic 35S_{pro}::*MsPOD* *Arabidopsis* plants obtained after selection were confirmed as transgenic plants expressing *MsPOD* by PCR, RT-PCR, and GUS staining (Figure 3A). The results show that four transgenic lines had high *MsPOD* expression levels, which were selected to produce seeds and for further analysis.

Figure 3. β -glucuronidase (GUS) expression and catalase (CAT) and peroxidase (POD) activity in transgenic and wild-type (WT) *Arabidopsis* plants. **A.** Representative GUS expression in 35S_{pro}::GUS transgenic and WT plants. **B.** CAT activity in transgenic and WT plants. **C.** POD activity in transgenic and WT plants. Error bars represent SEs (N = 4)

CAT and POD activity in transgenic plants

There was no distinguishable difference in CAT activity between transgenic *Arabidopsis* and WT plants (Figure 3B), but POD activity had increased in the transgenic *Arabidopsis* plants (Figure 3C). These results show that *MsPOD* expression in transgenic *Arabidopsis* plants only increased POD activity, indicating that *MsPOD* increases POD activity in transgenic *Arabidopsis* plants.

Expression of *MsPOD* enhances NaCl and H₂O₂ tolerance

Based on its expression patterns under H₂O₂ conditions, we supposed that *MsPOD* plays an important role in tolerance to abiotic stress. To assess H₂O₂ resistance in transgenic *Arabidopsis* plants, detached leaves were soaked in 3 mM 2-(N-morpholino) ethanesulfonic acid (MES) with the addition of 2.5 or 5% (v/v) H₂O₂ for three days. Leaf senescence was significantly delayed in 35S_{pro}::*MsPOD* transgenic plants, particularly with 5% H₂O₂ (Figure 4A). The WT leaves faded to yellow, but leaves from the 35S_{pro}::*MsPOD* transgenic plants were still partially green (Figure 4A). A similar pattern was observed of leaf chlorophyll content (Figure 4B). The chlorophyll content of the transgenic plants was much higher than that of the WT plants after treatment with H₂O₂ for three days, demonstrating that *MsPOD* can increase tolerance to H₂O₂.

Seeds of transgenic *Arabidopsis* and WT plants were sown in ½ MS plates with 0, 50, 100, or 150 mM NaCl to assess their salt resistance. After growing on plates for 15 days, the transgenic seedlings exhibited enhanced salt tolerance. With 50 mM NaCl, there was no obvious difference between WT and transgenic seedlings. The transgenic seedlings had longer roots than the WT seedlings after 100 or 150 mM NaCl was added to the ½ MS plates (Figure 4C), indicating that *MsPOD* expression in transgenic *Arabidopsis* plants enhanced resistance to NaCl.

Figure 4. H₂O₂ and NaCl incubation of *Arabidopsis MsPOD* transgenic plants. **A.** Detached third and fourth rosette leaves of wild-type (WT) and *MsPOD* transgenic plants were soaked in 3 mM MES with 2.5 or 5% (v/v) H₂O₂. The leaves were photographed on the third day. **B.** Measurement of the chlorophyll content of the detached leaves. **C.** Seedlings of transgenic and WT *Arabidopsis* plants were sown and grown on ½ Murashige and Skoog plates with 0, 50, 100, or 150 mM NaCl. **D.** Comparison of seedling root lengths in transgenic and WT *Arabidopsis* plants. Error bars represent SEs (N = 3).

DISCUSSION

PODs play multiple biological roles in plants, including lignin biosynthesis, suberization, indole-3-acetic acid catabolism, H₂O₂- and organic hydroperoxide-scavenging, growth regulation, and tolerance to biotic and abiotic stressors (Sanchez et al., 1983; Hiraga et al., 2001; Kim et al., 2004; Valério et al., 2004; Liu et al., 2005; Bae et al., 2011). It is very important to isolate and identify a novel POD gene from plants. In this study, we isolated and characterized a POD gene from alfalfa by the RT-PCR method. The coding domain sequence was 1062 bp in length, which encoded 353 amino acids. The phylogenetic analysis showed that MsPOD is more structurally related to the POD from *M. truncatula* and is less related to other homologs.

Expression analysis showed that *MsPOD* was most highly expressed in the stems and leaves (Figure 1C). This finding is in agreement with those from previous studies, demonstrating that the expression of many POD genes is tissue-specific (Wang et al., 2013; Yao et al., 2013). The relatively high *MsPOD* expression level in the stems and leaves suggests that *MsPOD* may be involved in defense responses in those organs. Previous studies have reported that POD genes play important roles in stress responses, and our expression analysis revealed that *MsPOD* transcription increases with H₂O₂ or NaCl addition (Figure 1D, F), suggesting that

MsPOD might play an important role in preventing damage caused by H_2O_2 and NaCl during growth, which is in agreement with results from previous studies (Choi and Hwang 2012; Yao et al., 2013). *CaPO2*, which is a POD gene from pepper, is strongly induced by drought and NaCl, with high expression levels being detected up to 25 h following treatment (Choi and Hwang 2012). The plant hormone ABA plays an important role in regulating and responding to abiotic stress by regulating genome-wide gene expression following drought, high salinity, and cold treatments. When alfalfa was exposed to 20 μ M ABA, *MsPOD* expression levels were not obviously affected after the first 6 h, but expression decreased after 24 h (Figure 1G). This suggests that *MsPOD* is not involved in the ABA signaling pathway. It seems probable that *MsPOD* accumulation occurs in response to H_2O_2 and NaCl to prevent damage.

We analyzed the subcellular localization of the MsPOD protein using SignalP 4.1 Server, which showed that the MsPOD protein appeared outside of cells. Therefore, we expressed the MsPOD-GFP fusion protein in onion epidermal cells to confirm the subcellular localization. As predicted, the GFP signal appeared around the onion epidermal cells (Figure 2), which demonstrated that the MsPOD protein was localized outside cells. A previous study reported the subcellular localization of a POD protein, *swpa4*, in sweet potato, and showed that the sweet potato POD was secreted into the extracellular space (Kim et al., 2008). The extracellular space between cells seems to be where the MsPOD protein responds to abiotic stress.

POD and CAT play important roles in decreasing oxidative damage. In the enzymatic system, SOD constitutes the first line of defense against ROS by dismutating O_2^- to H_2O_2 . Subsequently, H_2O_2 is broken down by CAT, POD, and APX (Chen et al., 2015). We found that POD levels had significantly increased in *MsPOD* transgenic *Arabidopsis* plants compared to those in WT plants, but CAT levels had not changed. Similarly, (Kim et al., 2008) reported that POD activity in *swpa4* transgenic plants was approximately 50-fold higher than that in control plants. POD activity in *CanPOD*-silenced plants was drastically reduced compared to that in control plants (Wang et al., 2013). These results indicate that the overexpression of *MsPOD* increases H_2O_2 tolerance in transgenic plants.

ROS are one of the most destructive substances to plants, and are generated under severe abiotic and biotic environmental conditions. PODs are thought to protect plants against oxidative stress. To date, many POD genes have been cloned and identified in various plant species, and many of them play important roles in abiotic or biotic resistance. For example, POD genes in wheat are involved in powdery mildew infection (Liu et al., 2005). Overexpression of a potato POD gene (*swpa4*) significantly increases salt- and drought-stress tolerance in transgenic plants (Kim et al., 2008). Overexpression of the pepper *CaPO2* gene in transgenic *Arabidopsis* plants not only increases tolerance to salt, drought, and oxidative stress, but also increases resistance to infection by the necrotrophic fungal pathogen, *Alternaria brassicicola* (Choi and Hwang 2012). Recently, (Wang et al., 2013) found that a novel *CanPOD* gene in pepper is involved in defense responses to *Phytophthora capsici* infection and abiotic stress tolerance. Based on the expression patterns of alfalfa *MsPOD* in response to H_2O_2 or NaCl addition, we suggest that *MsPOD* could decrease damage caused by abiotic stress. In addition, the expression of a tomato POD gene, *TPX2*, in transgenic tobacco increases germination rate under high-salinity conditions (Amaya et al., 1999). We found that tolerance to NaCl and H_2O_2 increased when *MsPOD* was expressed in transgenic *Arabidopsis* plants. After treatment with H_2O_2 for three days, *MsPOD* transgenic plants exhibited increased resistance to H_2O_2 and leaf senescence was delayed (Figure 4A). Chlorophyll content is also an important factor in the stress responses of plants; plants with high abiotic tolerance have

a high chlorophyll content (Sanchez et al., 1983; Bijanzadeh and Emam 2010). We found no significant difference in chlorophyll content between transgenic and WT leaves without H₂O₂, but it significantly decreased when treated with H₂O₂. The chlorophyll content of transgenic leaves was significantly higher than that of WT leaves, suggesting that *MsPOD* increased the H₂O₂ tolerance of the transgenic plants. Many genes that are involved in NaCl tolerance have been identified, and breeders have investigated the genetic mechanisms involved (Saeed et al., 2011). We also found that *MsPOD* is involved in NaCl resistance. Heterologous *MsPOD* expression improved the NaCl tolerance of transgenic plants treated with 100 or 150 mM NaCl compared to that of control plants. These results demonstrate that *MsPOD* increases resistance to H₂O₂ and NaCl.

We found that *MsPOD* expression increased abiotic stress tolerance. It appears that the involvement of *MsPOD* may be induced by abiotic stressors such as NaCl and H₂O₂, not via ABA or because of POD regulation. In conclusion, we isolated a POD gene named *MsPOD* from alfalfa, the expression of which was induced by NaCl and H₂O₂. The putative protein was localized outside of the cells. *MsPOD* expression in transgenic plants increased tolerance to NaCl and H₂O₂.

Conflicts of interests

The authors declare no conflict of interest.

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